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Measurement of Mitochondrial ATPase Activity in Maize Root Tips by Saturation Transfer ^{31}P Nuclear Magnetic Resonance[1]

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ABSTRACT

We show that saturation transfer ^{31}P nuclear magnetic resonance can be used to measure the activity of the mitochondrial ATPase of maize (*Zea mays* L. hybrid WW × Br38) root tips *in vivo*. Unidirectional rates of ATP synthesis were determined in the steady state (*i.e.* ATP and cytoplasmic orthophosphate constant) under various conditions. These measurements, and determinations of oxygen consumption, give a P/O ratio (measured in the living tissue) close to 3. In succinate-fed root tips the P/O ratio is approximately 2. Cyanide inhibits the rate of ATP synthesis by two-thirds (P/O ratio ~1), with an effective inhibitor constant of ~35 micromolars. We show that the alternative electron pathway cannot make ATP, and does not normally operate in this tissue. This method of studying plant mitochondrial metabolism avoids potential artifacts encountered in studies using isolated and purified mitochondria. The method also allows, for the first time, direct and simultaneous examination of the relationship between the rate of ATP synthesis and levels of metabolites such as ATP, and derived parameters such as phosphorylation potential.

A prerequisite for understanding regulation along or between metabolic pathways is the ability to accurately quantitate fluxes through individual pathways (2). The rates of mitochondrial electron transport can be estimated *in vivo* from measurements of O_2 consumption, assuming that oxidases other than Cyt oxidase and the alternative oxidase (3, 6, 7, 14) can be ignored. Currently, our concepts of how these estimated rates of electron transport relate to rates of ATP synthesis via oxidative phosphorylation have been based on *in vitro* experiments with isolated mitochondrial preparations. Two particular attributes of plant mitochondrial preparations make them less than ideal models of mitochondrial function *in vivo*. First, undamaged, pure plant mitochondria are more difficult to prepare than animal mitochondria (17), because of the large forces required to rupture plant cells, and possible inhibition of mitochondrial function by substances such as phenolics, or contamination with lipoxygenase (14). Despite considerable refinement of purification methods, plant mitochondria almost invariably show lower P/O ratios (mol ATP generated per O atom consumed) and, particularly, lower repiratory control ratios than animal mitochondria *in vitro* (17). Second, plant mitochondria possess a cyanide-insensitive electron transport pathway to an alternative oxidase; this pathway is generally found not to be capable of generating ATP *in vitro* (14, 16, 22). Since the relative contribution of the alternative and Cyt pathways to total electron transport (O_2 consumption) apparently varies not only from tissue to tissue (7), but also during aging and in response to factors such as ethylene (7, 14), it is difficult to relate rates of O_2 consumption to rates of ATP synthesis.

Saturation transfer ^{31}P NMR[3] has been used to study exchange of ^{31}P in a number of living systems (5, 10, 11; see Ref. 19 for review). The method permits determination of unidirectional reaction rates under steady state conditions *in vivo*. The method relies on the ability to specifically 'label' nuclear spins of a particular chemical group, such as the γATP phosphate, by saturating them with radiofrequency radiation of the appropriate frequency. Chemical reaction of this ATP will lead to transfer of these saturated spins to the spin populations of other chemical groups, such as cytoplasmic P_i. This transfer will result in a decrease in the intensity of the P_i resonance, provided the reaction rate is comparable to the rate at which the label decays (characterized by T_1, the longitudinal relaxation time of the P_i resonance). The magnitude of the saturation transfer effect provides information on the unidirectional rate of reaction. We show here that this method can be used to determine rates of ATP synthesis catalyzed by the mitochondrial ATPase in living maize root tips. From such measurements we can evaluate the efficiency of electron transport under a wide range of conditions.

MATERIALS AND METHODS

Maize (*Zea mays* L.) hybrid WW × Br38 (Customaize Research, Decatur, IL) were grown for 2 d in the dark. Root tips, 1.5 to 2 mm long, were excised, rinsed with and stored in 50 mM glucose plus 0.1 mM $CaSO_4$. Root tips were perfused as described prevously (20); perfusion media were saturated with O_2 (or for hypoxic treatments, N_2), and bubbling was continued throughout the experiment, except for treatments involving KCN in which the O_2-saturated solution was maintained under an atmosphere of pure O_2.

O_2 consumption by perfused maize root tips was determined polarographically, using a Harvard Apparatus (South Natick, MA) O_2 electrode, and amplifier to measure the concentration of O_2 before and after the sample (Fig. 1), knowing the tissue fresh weight, perfusion rate, and the solubility of O_2 (24).

^{31}P NMR spectra were obtained using a modified Bruker HXS-360 spectrometer, operating at 145.78 MHz in the Fourier transform mode. Measurements of saturation transfer were made by standard experimental procedures (11), as follows. The resonance of interest (γATP, or cytoplasmic P_i), was irradiated with suffi-

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[3] Abbreviations: NMR, nuclear magnetic resonance; SHAM, salicylhydroxamic acid; K_i inhibitor constant; $FADH_2$, flavin-adenine dinucleotide (fully reduced form).

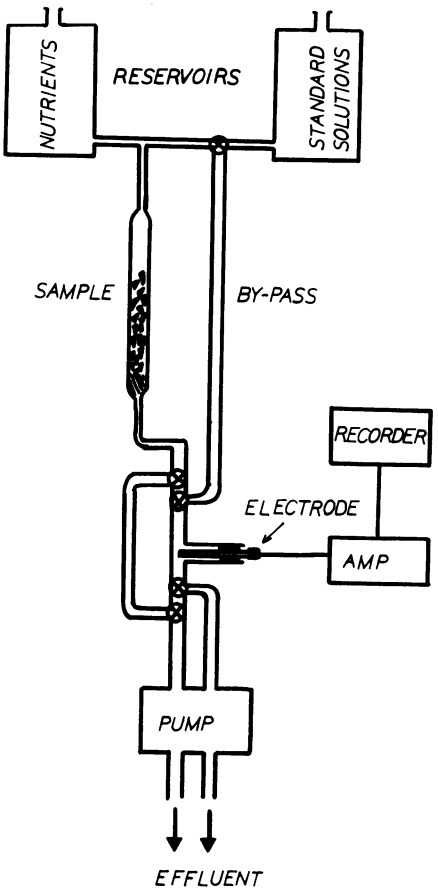

FIG. 1. Schematic diagram of apparatus used to determine O_2 consumption in perfused maize root tips. Stop-cocks were adjusted so the O_2 electrode measured the O_2 tension either before or after the sample, from which O_2 consumption was calculated (see "Materials and Methods"). The electrode could be calibrated with standard solutions, permitting correction for any drift occurring over the long (10 or more h) experiments.

cient radiofrequency power to just saturate it, so the peak disappeared from the spectrum; the selective irradiation was gated such that it was off during spectral acquisition. The spectrum obtained under these conditions (spectrum 1) was compared with a spectrum obtained while irradiating the spectral region equidistant from the resonance showing saturation transfer (spectrum 2). The area of this resonance in spectrum 1 was divided by its area in spectrum 2 to give M^*, the magnitude of the saturation transfer effect. These two spectra were generated by adding together 32 scan blocks, the selective irradiation changing after each block. Normally, scans were made every 2.4 s, with a spectral width of ±9000 Hz and 8K data points; 15-Hz linebroadening was applied to spectra prior to examination, by exponential multiplication of the free induction decay prior to Fourier transformation. Saturation transfer measurements were made over periods ranging from 1 h (sufficient for measurement of ATP synthesis rates in oxygenated, glucose-fed root tips) to more than 12 h (for measurements in cyanide treated, or hypoxic tissues). Longitudinal relaxation times (T_1), required for calculation of exchange rates, were measured by the saturation recovery method (12). The absolute concentrations of cytoplasmic P_i and ATP were determined by comparing areas of peaks in root tip spectra obtained under nonsaturating conditions (30-s pulse interval), with peak areas of standard phosphate solutions; areas were compared by including a reference capillary containing 0.5 M methylene diphosphonate (pH 8.9 in Tris) in both samples. Assignments of peaks in maize root tip ^{31}P NMR spectra (18) are given in the figure legends.

Exchange of ^{31}P between cytoplasmic P_i and γATP in maize root tips was considered as a simple exchange reaction:

$$ATP + H_2O \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ADP + P_i + H^+ \quad (1)$$

with k_1 specifying the rate constant for ATP hydrolysis (due to biosynthesis and solute transport), and k_2 specifying the rate constant for ATP synthesis (k_1 and k_2 are equal to the reciprocals of the average lifetimes of ATP and P_i, respectively). All experiments described here were performed in the steady state, except for the succinate experiments (where ATP levels increased gradually, at a rate < 1/20 the estimated unidirectional rate of ATP synthesis) (Fig. 10), and immediately after treatment of root tips with 0.5 mM KCN (Fig. 8). In the steady state:

$$\text{Rate of ATP synthesis} = k_1\,[ATP] = k_2\,[P_i] \quad (2)$$

If this simple description applies to maize root tips, then k_2 can be calculated from saturation transfer data, such as that in Table I, using the following equation ([9]; for a useful account of saturation transfer theory applied to simple exchanging systems see [11]):

$$k_2 = \frac{1}{T_1\,M^*} - \frac{1}{T_1} \quad (3)$$

where M^* is the intensity (area) of the cytoplasmic P_i resonance with the γATP resonance saturated, as a fraction of its intensity with the γATP resonance unsaturated (determined as described above), and T_1 is the longitudinal relaxation time of the cytoplasmic P_i resonance in the absence of chemical exchange, given by:

$$\frac{1}{T_1'} = \frac{1}{T_1} + k_2 \quad (4)$$

where T_1' is the longitudinal relaxation time of the cytoplasmic P_i resonance measured in the presence of selective γATP saturation. Knowing M^* and T_1', k_2 can be obtained by solving simultaneous equations 3 and 4. The rate of ATP synthesis is given by insertion of this value of k_2, together with the tissue concentration of cytoplasmic P_i, into equation 2.

Analogous expressions apply to the determination of k_1, from experiments in which the cytoplasmic P_i resonance is saturated. These equations assume that there is complete relaxation of spins between pulses; this condition was essentially met in the experiments reported in this paper: increasing the pulse interval from 2.4 to 30 s resulted in no statistically significant decrease in M^* for oxygenated, glucose perfused root tips.

RESULTS

Observation of Saturation Transfer in Glucose-Perfused Maize Root Tips. Transfer of saturation from the ^{31}P NMR γATP resonance to the cytoplasmic P_i resonance in oxygenated, glucose-perfused maize root tips is shown in Figure 2: irradiation of the γATP resonance results in a 17.5% reduction in the intensity of the cytoplasmic P_i resonance, *i.e.* M^* is 0.825 (Table I, first line). T_1' for cytoplasmic P_i was found to be 1.35 s, so that T_1 is 1.6364 ± 0.01 s. From these measured values, and using the analytical approach outlined in "Materials and Methods," k_2 and the ATP synthesis rate can be determined for root tips under these conditions (Table I, line 1). Comparison of the determined rate of ATP synthesis with rates of O_2 consumption measured as described in "Materials and Methods" allows direct observation, in living root tips, of the efficiency of mitochondrial electron transport; a P/O ratio close to 3 was found in aerobic glucose-

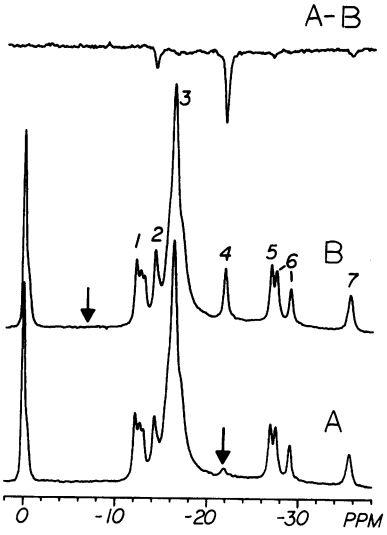

FIG. 2. Saturation transfer from γATP to cytoplasmic P_i in aerobic maize root tips. Saturation transfer spectra obtained with root tips perfused with O_2-saturated 50 mM Glc + 0.1 mM $CaSO_4$, 40 ml/min. Spectrum A obtained with selective presaturation of the γATP resonance; spectrum B obtained with selective irradiation of a point equidistant from the cytoplasmic P_i line (peak 2) on the low field side of the spectrum (as indicated by arrows). Spectrum A-B is the difference between spectrum A and spectrum B, showing the transfer of saturation from the γATP line to the cytoplasmic P_i line. Spectra were obtained over a period of about 6 h. Peak assignments: 1, Glc-6-P; 2, cytoplasmic P_i; 3, vacuolar P_i; 4, γATP; 5, αATP; 6, UDPG + nicotinamide adenine nucleotides; 7, βATP. The two 'shoulders' to the right of 1 (well resolved in Fig. 9) are unassigned.

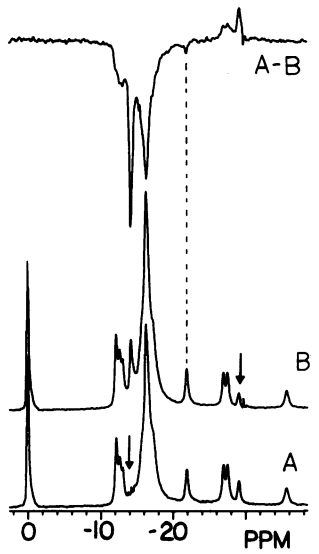

FIG. 3. Saturation transfer from cytoplasmic P_i to γATP in aerobic maize root tips, perfused as in Figure 1. Spectrum A obtained with selective presaturation of the cytoplasmic P_i resonance; spectrum B obtained with selective irradiation of the point equidistant from the γATP line on the high field side of the spectrum (as indicated by arrows). Spectrum A-B is the difference between spectrum A and spectrum B, showing the transfer of saturation from the cytoplasmic P_i line to the γATP line. Spectra were obtained over 8 h.

fed root tips (Table I).

The reciprocal saturation transfer effect, from cytoplasmic P_i to γATP, can also be observed (Fig. 3), although a large range of values for M^* (0.85–0.96) was found. This is attributed to the very short T_1 of the γATP resonance (0.37 ± 0.05 s), which consequently dominates the expression for k_1 (*cf.* equation 3 in "Materials and Methods"), such that M^* is relatively small. These values for T_1 and M^* lead to a calculated rate constant for ATP utilization, k_1, of 0.325 ± 0.225 s^{-1}. The intracellular concentration of ATP under these conditions was found to be 0.528 ± 0.05 mM. Hence, the rate of ATP hydrolysis determined by saturation transfer is 0.1716 ± 0.13 $\mu M \cdot g^{-1} \cdot s^{-1}$, similar in magnitude to 0.1426 ± 0.019 $\mu M \cdot g^{-1} \cdot s^{-1}$, the measured rate of ATP synthesis (Table I, line 1). This result suggests that analysis of the saturation transfer data as a simple exchange reaction is valid, although the error in this comparison is significant. Other potential objections to this analysis of the saturation transfer data are considered in the "Discussion".

In anaerobic maize root tips, saturation transfer from γATP to cytoplasmic P_i cannot be observed (*i.e.* transfer is reduced below the level of detection) (Fig. 4; Table I). This inhibition of saturation transfer under hypoxia is relieved on reoxygenation (data not shown). These results indicate that the saturation transfer (Fig. 2) is due to an O_2-dependent phosphoryl exchange reaction, most likely oxidative phosphorylation (ATP synthesis) and biosynthesis (ATP utilization).

Effect of Exogenous Glucose. O_2 consumption in maize root tips perfused with 0.1 mM $CaSO_4$ increases by approximately 50% following perfusion with 50 mM glucose (Table I). This increase in O_2 consumption is matched by an increase in the magnitude of the saturation transfer effect from γATP to cytoplasmic P_i (Table I). Table I and Figure 5 show that cytoplasmic P_i levels are higher in the less metabolically active, glucose-deprived maize root tips. These results indicate that the efficiency of oxidative phosphorylation (P/O ratio) is unaffected by exogenous glucose (Table I). ATP levels are essentially unchanged by the glucose treatment (Fig. 5). The higher cytoplasmic P_i levels observed in glucose-deprived root tips are associated with lower glucose-6-P and UDPG levels (Fig. 5).

Effect of Cyanide and SHAM. Perfusion of glucose-fed root tips with 0.5 mM KCN causes a rapid inhibition of O_2 consumption (Table I). However, after a period of hours, the rate of respiration increases to levels close to that in cyanide-free tissue (Table I), *i.e.* their respiration becomes almost completely cyanide resistant. Perfusion with fresh cyanide solution resulted in no change in respiration rate, indicating that the recovery was not due to loss of cyanide from the reservoir by volatilization. Essentially no inhibition of O_2 consumption was observed in tissue exposed to 0.05 mM KCN (Table I).

The inhibition of ATP synthesis by increasing various concentrations of KCN, determined from saturation transfer data, is shown in Figure 6. The data were obtained under conditions where O_2 consumption was essentially constant (within 10%), *i.e.* at high cyanide concentrations (0.25 and 0.5 mM) measurements were made after the initial, dramatic respiratory inhibition had subsided. Increasing the concentration of cyanide results in a monotonic decrease in the saturation transfer effect, to one-third of the effect seen in untreated root tips, with an effective K_i of approximately 35 μM. Thus, cyanide reduces the efficiency of respiration (P/O ratio) from approximately 3 to approximately 1 (Table I).

Although both cyanide and hypoxia drastically reduce rates of ATP synthesis (Table I), the two treatments have quite different effects on the concentrations of cytoplasmic phosphates, as determined by peak areas in ^{31}P NMR spectra of root tips. In hypoxia, ATP and glucose-6-P decrease, while cytoplasmic P_i increases (Figs. 2 and 4); cyanide causes an increase in glucose-6-P, while essentially no change in cytoplasmic P_i occurs (Fig. 7; Table I), except for a transient increase following treatment with 0.5 mM KCN (Fig. 8; Table I). Only when cyanide (0.25 mM) is used in combination with 2 mM SHAM, *i.e.* when both terminal

Table I. *Effect of Various Treatments on Oxygen Consumption, Magnitude of the Saturation Transfer Effect from γATP to Cytoplasmic P_i (M^*), Tissue Cytoplasmic P_i Concentration, and the Derived Parameters: Rate of ATP Synthesis and P/O ratio*

Treatment			Response				
Exogenous respiratory substrate	Oxygen tension	Inhibitor	Rate of oxygen consumption (±SD)[a]	M^* (±SE)	Cytoplasmic $[P_i]$ (±SD)	Rate of ATP synthesis (range[b])	P/O ratio (range[b])
	% saturation		*μl g^{-1} h^{-1}*		*mM*	*μmol $g^{-1} \cdot s^{-1}$*	
Glc, 50 mM	100	None	1800 (±100)	0.825 (±0.005)	1.1 (±0.1)	0.1426 (0.1244–0.1620)	3.19 (2.64–3.67)
Glc, 50 mM	0	None	0	1	2.6 (±0.1)	<0.02[c]	
None	100	None	1200 (±100)	0.92 (±0.01)	1.7 (±0.1)	0.093 (0.0731–0.1095)	3.03 (2.26–4.0)
Glc, 50 mM	100	0.05 mM KCN	1800 (±100)	0.9023 (±0.01)	1.1 (±0.1)	0.0728 (0.0579–0.0893)	1.63 (1.23–2.12)
Glc, 50 mM	100	0.5 mM kCN 0–2 h	1100 (±100)	0.98 (±0.002)	2.1 (±0.1)	0.0262 (0.0227–0.0304)	0.96 (0.83–1.11)
Glc, 50 mM	100	0.5 mM KCN after 4 h	1600 (±100)	0.942 (±0.003)	1.1 (±0.1)	0.0414 (0.0354–0.0479)	1.04 (0.84–1.29)
Glc, 50 mM	100	0.5 mM KCN + 2 mM SHAM	300 (±100)	1	2.6 (±0.1)	<0.02[c]	
Glc, 50 mM	100	2 mM SHAM	1700 (±100)	0.831 (±0.005)	1.1 (±0.1)	0.1367 (0.1192–0.1554)	3.24 (2.66–3.92)
Succinate, 50 mM	100	None	1300[d] (±100)	0.914 (±0.01)	1.1 (±0.1)	0.0632 (0.05–0.078)	1.96 (1.44–2.64)
Succinate, 50 mM	100	0.5 mM KCN	800 → 200	1	1.7 (±0.1)		

[a] Multiply by 2.48×10^{-5} to convert to μmol 0 atom $\cdot g^{-1} \cdot s^{-1}$.
[b] Range obtained by inserting extreme combinations of M^*, $[P_i]$, and T_1 into equations 2 and 3.
[c] Rate estimated from rate of ethanol fermentation assuming 1 ATP produced per ethanol molecule (data not shown).
[d] Decreases after ~4 h.

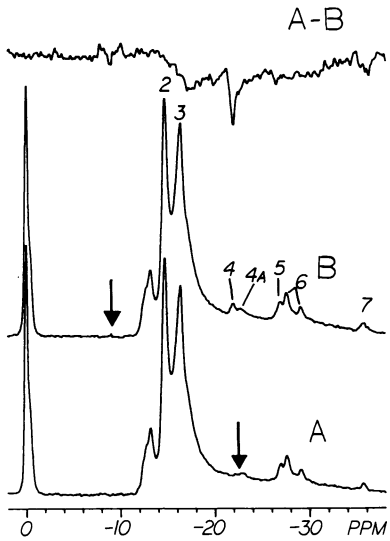

FIG. 4. Inhibition of saturation transfer from γATP to cytoplasmic P_i in maize root tips during hypoxia. Spectra were obtained with root tips perfused with N_2-saturated 50 mM Glc + 0.1 mM $CaSO_4$, at 5 ml/min. Spectrum A obtained with selective presaturation of the γATP line; spectrum B is the 'control', obtained with irradiation of the spectral region equidistant from the cytoplasmic P_i line on the low field side. Spectrum A-B is the difference between spectrum A and spectrum B, showing no transfer of saturation from γATP to cytoplasmic P_i. Assignment of peaks is given in the legend to Figure 2, except peak 4A: βADP. Spectra were obtained over 12 h.

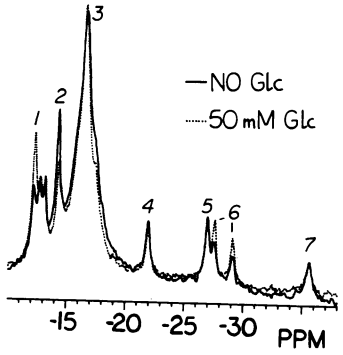

FIG. 5. Effect of glucose on ATP, cytoplasmic P_i, UDPG, and Glc-6-P concentrations in maize root tips. ^{31}P NMR spectra of perfused, aerobic root tips, were obtained in approximately 1 h. (——), The sample perfused with oxygenated 0.1 mM $CaSO_4$; (– – –), the same sample, but 30 min after making the perfusion medium 50 mM Glc. Assignments given in Figure 2 legend.

oxidases of the mitochondrial electron transport chain are inhibited, is a spectrum virtually identical to that of hypoxic root tips obtained (Fig. 7). This combination of inhibitors also causes complete inhibition of saturation transfer from γATP to cytoplasmic P_i ($M^* = 1$ in Table I) and almost complete inhibition of O_2 consumption—behavior analogous to hypoxia.

O_2 consumption, ATP synthesis rate, and the efficiency of respiration are almost unaffected by exposure to 2 mM SHAM alone (Table I), as are concentrations of cytoplasmic phosphates (Fig. 7). One interesting effect of SHAM was observed, however; it caused a considerable decrease in the line width of the vacuolar P_i resonance (Figs. 7 and 9). This narrowing took place over a period of hours (complete within approximately 5 h), and par-

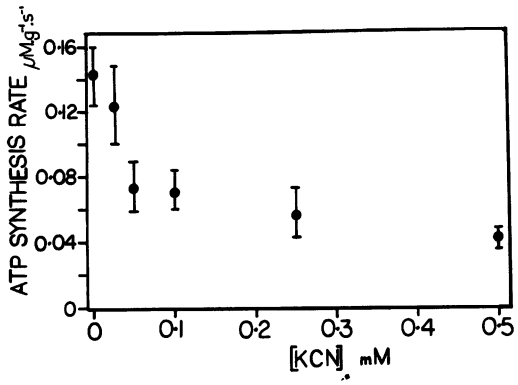

FIG. 6. The inhibition of ATP synthesis is perfused, aerobic maize root tips by cyanide. ATP synthesis was measured by saturation transfer NMR as described in "Materials and Methods." Error bars represent standard deviations from at least 4 separate measurements. The measurements were made under conditions where O_2 consumption was essentially constant (see text).

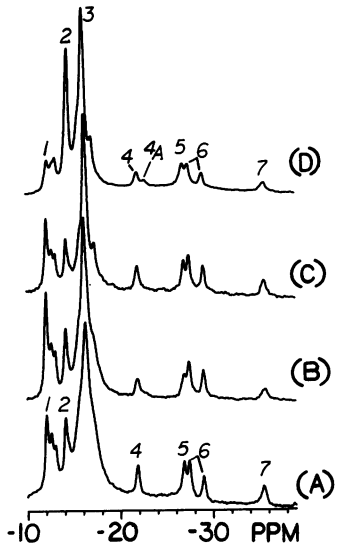

FIG. 7. Effect of KCN and SHAM on concentrations of phosphates in maize root tips. ^{31}P NMR spectra of oxygenated root tips perfused with: A, 50 mM Glc + 0.1 mM $CaSO_4$; B, same as A + 0.5 mM KCN pH 7; C, same as A + 2 mM SHAM pH 7; D, same as A + 0.5 mM KCN + 2 mM SHAM, pH 7. Assignments as in Figure 2 except 4A: βADP. Spectra were each obtained over approximately 2 h.

alleled the increasing effect of SHAM over time (*e.g.* the effect of SHAM on respiration in cyanide-treated root tips), indicating slow penetration/uptake of this compound by the tissue. The narrowing effect is almost certainly due to the ability of SHAM to complex paramagnetic ions present in the vacuole, and so stop these ions from broadening the P_i resonance. This view is supported by the observation that an increase in the T_1 of the vacuolar resonance accompanies the line narrowing (paramagnetic agents commonly result in very short relaxation times) (data not shown); moreover, Kime *et al.* (13) have shown that paramagnetic species such as Mn^{2+} are preferentially sequestered in the vacuoles of maize root tips following uptake, this leading to increased and selective broadening of the vacuolar P_i resonance. It appears, then, that chemical shift heterogeneity, due to P_i in different vacuoles being present in different environments, such as pH, cannot be the principal cause of the broad vacuolar P_i peak seen in maize root tips, as we previously postulated (20). The dramatic decrease in line width induced by SHAM enables

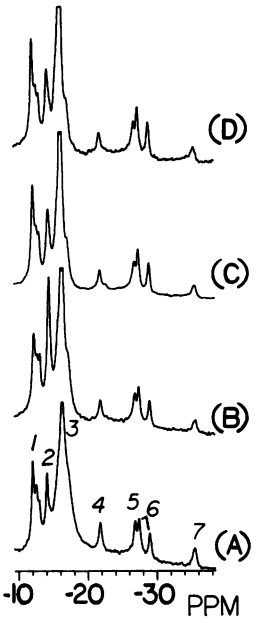

FIG. 8. Effect of 0.5 mM KCN on cytoplasmic P_i levels in maize root tips. ^{31}P NMR spectra of a single sample of oxygenated root tips initially perfused with 50 mM Glc + 0.1 mM $CaSO_4$ (A). Spectrum B was obtained during the first 2 h after addition of 0.5 mM KCN pH 7; note the increase in cytoplasmic P_i (peak 2). Spectrum C was obtained 4 to 8 h after KCN addition; note the decrease in cytoplasmic P_i. Spectrum D was obtained during the first 2 h after perfusion with a fresh 0.5 mM KCN solution; no change relative to spectrum C is apparent.

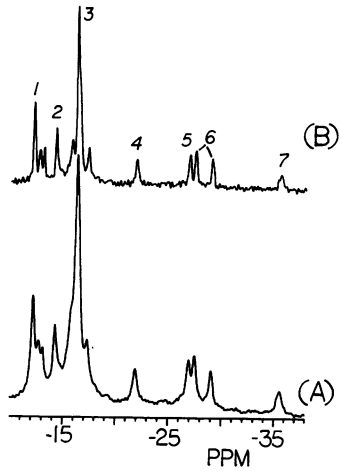

FIG. 9. Improved spectral resolution in maize root tips treated with SHAM. ^{31}P NMR spectra of maize root tips perfused with oxygenated 50 mM Glc + 0.1 mM $CaSO_4$ + 2 mM SHAM (4 h after addition of SHAM). Spectrum A is a 'normal' spectrum, obtained using line broadening of 15 Hz; B is a resolution-enhanced spectrum, obtained from the same accumulated free induction decay used to generate spectrum A. The resolution enhancement (using double exponential multiplication [8]) gives clear definition of two peaks, one on each side of the vacuolar P_i resonance (peak 3). These resonances, and the two lines to the right (upfield) of the Glc-6-P resonance (peak 1) are at present unassigned. Other assignments are given in Figure 2.

two, as yet unassigned, phosphate signals to be clearly resolved (Fig. 9).

Effect of Succinate and Cyanide. When root tips are provided with 50 mM succinate as respiratory substrate, instead of glucose, lower rates of respiration and ATP synthesis are observed, and

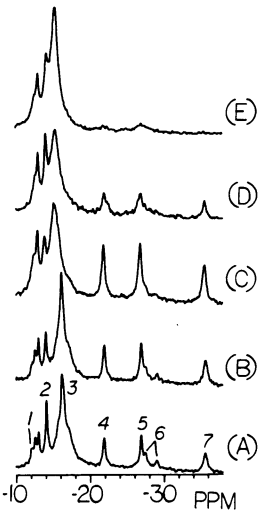

FIG. 10. The effect of succinate, and succinate + 0.5 mM KCN on ATP levels in perfused maize root tips. Sequential ^{31}P NMR spectra of a single root tip sample perfused initially with O_2-saturated 0.1 mM $CaSO_4$ (A); 1 to 3 h of perfusion with 50 mM succinate (to pH 7 with Tris base) (B); 4 to 6 h of perfusion as in B (C); 0.5 to 2 h of perfusion with 50 mM succinate + 0.5 mM KCN pH 7 (D); 2 to 4 h of perfusion as in D (E). Assignments are given in Figure 2 legend.

the P/O ratio is closer to 2 than 3 (Table I). This treatment causes a remarkable increase in ATP levels, as glucose-6-P and UDPG virtually disappear (Fig. 10). If 0.5 mM KCN is then included in the perfusion medium, the rate of ATP synthesis falls below that detectable by saturation transfer (in our present experimental arrangement) (Table I) and ATP disappears after 2 h of KCN treatment (Fig. 10).

DISCUSSION

The Saturation Transfer Effect from γATP to Cytoplasmic P_i Is Catalyzed by Mitochondrial ATPase. Although innumerable reactions are known by which ^{31}P can exchange between ATP and P_i in the cell, it is clear from our results that the principal reaction responsible for the transfer of saturation from γATP to cytoplasmic P_i is mitochondrial oxidative phosphorylation, for the following reasons. First, the saturation transfer effect from the γATP to cytoplasmic P_i is abolished when root tips become hypoxic (Fig. 4), *i.e.* when mitochondrial electron transport ceases. Second, the changes in the magnitude of the saturation transfer effect parallel the changes in rates of O_2 consumption seen when glucose is added to maize root tips (Table I). Third, the saturation transfer effect is not observed in root tips treated with inhibitors of both mitochondrial electron transport terminal oxidases, *viz.* cyanide (inhibitor of Cyt oxidase) plus SHAM (inhibitor of the alternative oxidase) (Table I). Fourth, and finally, the K_i for cyanide inhibition of the saturation transfer effect (Fig. 6) is similar in magnitude to the K_i for cyanide inhibition of respiration in fresh potatoes (which lack the alternative electron pathway) (23), and to the binding constant of cyanide for Cyt oxidase *in vitro* (25).

The Precision, Accuracy, and Validity of Measurements of ATP Synthesis Rates by Saturation Transfer ^{31}P NMR. Although analysis of the saturation transfer data presented in this paper, using the approach outlined in "Materials and Methods," gives readily interpretable conclusions, it is important to point out that it is impossible to rigorously show that this method of analysis is valid, if only because we know very little about phosphate exchange reactions in intact tissues. The essential problem is that the NMR experiment provides only one measurable parameter, M^*, which gives no information on the kinds or numbers or relative rates of reactions responsible for the observed transfer of saturation. Validation of the technique will only be achieved by demonstrating that it correctly predicts significant physiological phenomena.

Assuming that the method of analysis is valid, the saturation transfer method appears capable of quantitating changes in rates of ATP synthesis, and the efficiency of oxidative phosphorylation, in maize root tips (Table I). Note that the estimates of uncertainty in the determined values of these parameters (last two columns, Table I; Fig. 6) are conservative, coming from insertion into equations 2 and 3 of extreme combinations of standard deviations or standard errors of M^*, T_1, cytoplasmic P_i concentration, and O_2 consumption (Table I).

Another aspect of ATP synthesis rate measurements relates to potential systematic error due to systematic under- or overestimation of the intensity of the P_i resonance. This error may potentially arise from the lack of complete resolution of the cytoplasmic P_i resonance (Fig. 2), so its true intensity (peak area) might be systematically under- or overestimated, leading to systematic under- or overestimates of M^* and $[P_i]$. Here we describe quantitatively how such errors would affect the accuracy of our rate measurements.

If intensity of the cytoplasmic P_i resonance is systematically under- or overestimated, both $[P_i]$ and M^* will be systematically under- or overestimated, the latter according to:

$$M^*_{OBS} = \frac{M_* + \frac{[P_i^{TRUE}] - [P_i^{OBS}]}{[P_i^{TRUE}]}}{1 + \frac{[P_i^{TRUE}] - [P_i^{OBS}]}{[P_i^{TRUE}]}} \quad (5)$$

where $[P_i^{OBS}]$ and $[P_i^{TRUE}]$ are the observed (apparent) and true cytoplasmic P_i concentrations, respectively. The error in the calculated ATP synthesis rate resulting from insertion of M^*_{OBS} into equation 3, and $[P_i^{OBS}]$ into equation 2 is shown in Figure 11; it is apparent that significant (>20%) error in estimation of ATP synthesis rate arises only when $[P_i]$ is underestimated by

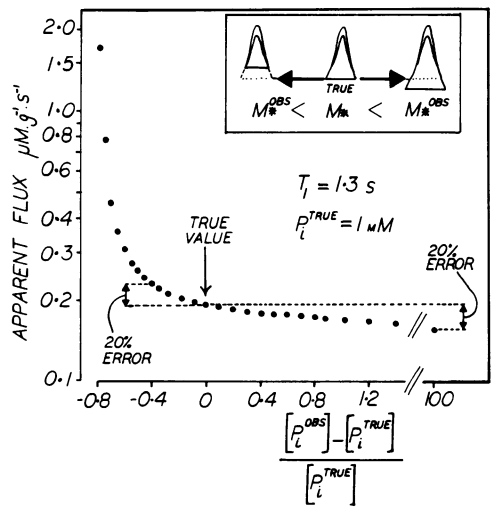

FIG. 11. Quantitation of potential error in saturation transfer measurements of rates of ATP synthesis, due to errors in determination of the intensity of the cytoplasmic P_i line. The figure shows how M^* is under- or overestimated when the intensity of the P_i resonance is under- or overestimated, respectively (see inset). Such under- or overestimations of M^* and P_i lead to over- or underestimations, respectively, of the rate of ATP synthesis. The graph represents errors assuming T_1 for the cytoplasmic P_i resonance is 1.3 s, $M^* = 0.8$ (true value), and the true cytoplasmic P_i concentration is 1 mM.

40% or more, an error certainly not applicable to our estimate of [P_i]. Hence, this potential source of uncertainty can be considered to be insignificant.

The Efficiency of Mitochondrial Electron Transport, and the Contribution of the Alternative Electron Pathway to ATP Synthesis. Maize root tip respiration is dependent upon exogenous sugar (21), normally provided by the caryopsis or leaves. When deprived of sugar, root tip respiration declines as endogenous carbohydrates are depleted (21). In both cases, NADH is the principal electron donor to the mitochondrial electron transport chain. In carefully prepared, intact plant mitochondria each NADH gives rise to three molecules of ATP (3). This accounts for the observed P/O ratio of approximately 3 found in both glucose-perfused and 'starved' maize root tips (Table I). Clearly oxidative phosphorylation is operating at, or very near to, full efficiency, *i.e.* mitochondria are 'tightly coupled' *in vivo*. Similar P/O ratios, also determined by a combination of saturation transfer NMR and O_2 consumption measurements, have been found in rat heart (15) and rat kidney (10). However, in a study of yeast cells (in stationary phase, in the absence of exogenous glucose) (1), such an experiment led to a P/O ratio of nearly 90, a thermodynamically impossible efficiency. This high P/O ratio was attributed to rapid exchange of ^{31}P between P_i and ATP catalyzed by the mitochondrial ATPase (1), a result analogous to the rapid exchange of $^{32}P_i$ with the phosphate groups of adenine nucleotides seen in isolated mitochondria (*e.g.* 4). In other words, in these yeast cells, the principal consumer of ATP was the mitochondria ATPase, rather than biosynthetic, transport, and catabolic ATP-consuming reactions, in accord with the fact that these cells were not growing and lacked a carbon source. The observation of lower P/O ratios in root tips, hearts (15), and kidneys (10), tissues that are doing work, indicates that ATP-consuming reactions other than the mitochondrial ATPase dominate in ATP consumption. However, these lower P/O ratios do not, in themselves, preclude the possibility that the mitochondrial ATPase contributes to ATP consumption in these tissues, a possibility that would result in overestimation of the true efficiency of oxidative phosphorylation. Evidence against such an overestimation comes from the observation that lower P/O ratios were observed in succinate-fed tissue compared to glucose-fed tissue (Table I). ATP levels are much higher in the succinate-fed root tips (Figs. 2 and 10), and so hydrolysis of ATP by the mitochondrial ATPase is more favorable in this tissue. Thus, one would anticipate an increase in the rate of this reaction, and so an increase in the observed P/O ratio, with increasing ATP concentration, the opposite of the observed result. This finding suggests, therefore, that the P/O ratios calculated in this paper do indeed represent the efficiencies of oxidative phosphorylation in maize root tips.

If root tips are depleted of endogenous carbohydrate, and then provided with succinate, $FADH_2$ will be the principal electron donor to the mitochondrial electron transport chain. Each molecule of $FADH_2$ generated by succinate can give rise to two molecules of ATP in undamaged plant mitochondrial preparations (3). This accounts for the P/O ratio of approximately 2 found in succinate-fed maize root tips *in vivo* (Table I).

Half-millimolar cyanide causes the P/O ratio of glucose-fed root tips to fall from 3 to 1, while O_2 consumption remains essentially unchanged (Table I). It is well established that during cyanide resistant respiration electrons travel to the alternative terminal oxidase from a point between the first and second proton translocation sites of the Cyt pathway (7, 14, 16, 22), such that only the first proton translocation site of the Cyt pathway is used. The observation of a P/O ratio of 1 is most readily explained by considering that it is only this first proton translocation site, and no component of the alternative electron transport pathway, that drives ATP synthesis in cyanide-treated root tips, as has been inferred from *in vitro* experiments (7, 16, 22). This conclusion is supported by the observation that succinate-fed maize root tips treated with 0.25 or 0.5 mM KCN, in which only the alternative electron transport pathway can be expected to operate, cannot make ATP (Table I; Fig. 10).

It appears that the alternative electron transport pathway does not operate in root tips not treated with cyanide. Thus, a P/O ratio of 3 is observed in glucose-fed root tips, and this ratio is not significantly affected by exposure to 2 mM SHAM (Table I).

CONCLUSIONS

We have demonstrated that ^{31}P NMR can be used to directly measure rates of ATP synthesis catalyzed by mitochondrial in living maize root tips. These measurements permit direct determination of the efficiency of oxidative phosphorylation. The method offers a means of studying mitochondria in their natural environment, so that no artifacts due to damage during isolation have to be considered. This represents a considerable advantage, particularly as plant mitochondrial preparations often exhibit P/O ratios lower than most animal mitochondria. The method will for the first time enable direct and simultaneous examination of the relationship between rates of ATP synthesis, and levels of metabolites such as ATP, and derived parameters such as phosphorylation potential.

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